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This proposal requests funds to further involve graduate students in environmental research sponsored by the United States Air Force Office of Scientific Research (AFOSR). The participants will learn to utilize a combination of laboratory and field approaches to identifying physical, chemical, genetic, and physiological influences that govern the accumulation and biodegradation of polycyclic aromatic hydrocarbons (PAHs). These and related compunds are among the chemicals whose environmental fate is of concern to the U.S. Air Force and other Department of Defense agencies. The Principal Investigator and colleagues have conducted a prior, independent study that has shown that, despite the presence of PAH metabolizing microorganisms, PAHs persist at a site where freshwater sediments are fed by PAH-contaminated groundwater. Hypotheses to be tested address fundamental mechanisms for the persistence of environmental pollutants, these include: (1) the rate of delivery meets or exceeds the rate of biodegradation; (2) The PAHs are not available to microbial populations due to rapid, short term sorption onto the sediment organic matter, or due to long term (aging) sorption into a spatially remote compartment of the microporous structure of sediment organic matter, or due to complexation reactions with dissolved organic carbon, or due to the physical arrangement of the sediment matrix which prevents contact betw-en PAHs and microorganisms.

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First Annual Report

For AASERT grant #93-NL-073

entitled

"Research Training for Understanding the Fate of Environmental Pollutants"

submitted to:

Dr. Walter J. Kozumbo

Project officer at AFOSR

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submitted by:

Eugene L. Madsen

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Section of Microbiology

Cornell University

Ithaca, NY 14853

607-255-3088

June 30, 1994

The objectives of this AASERT grant are perhaps best appreciated by recalling statements in the Abstract of the original proposal which appears below:

ABSTRACT: This proposal requests funds to further involve graduate students in environmental research sponsored by the United States Air Force Office of Scientific Research (AFOSR). The participants will learn to utilize a combination of laboratory and field approaches to identifying physical, chemical, genetic, and physiological influences that govern the accumulation and biodegradation of polycyclic aromatic hydrocarbons (PAHs). These and related compounds are among the chemicals whose environmental fate is of concern to the U.S. Air Force and other Department of Defense agencies. The Principal Investigator and colleagues have conducted a prior, independent study that has shown that, despite the presence of PAH metabolizing microorganisms, PAHs persist at a site where freshwater sediments are fed by PAH-contaminated groundwater. Hypotheses to be tested address fundamental mechanisms for the persistence of environmental pollutants, these include: (1) the rate of delivery meets or exceeds the rate of biodegradation; (2) the PAHs are not available to microbial populations due to rapid, short term sorption onto the sediment organic matter, or due to long term (aging) sorption into a spatially remote compartment of the microporous structure of sediment organic matter, or due to complexation reactions with dissolved organic carbon, or due to the physical arrangement of the sediment matrix which prevents contact between PAHs and microorganisms; (3) the microorganisms may be physiologically limited by the presence of preferred metabolic substrates or toxic or inhibitory substances, or by the lack of proper final electron acceptors, electron donors, or inorganic or organic nutrients; and (4) PAHs may persist simply due to restricted distribution and abundance of biodegradation genes in naturally occurring microbial populations. By working in an iterative manner between field observations and controlled laboratory determinations, this research project will systematically test the above hypotheses and thus identify constraints on microbiological processes that destroy PAHs (naphthalene and phenanthrene). The graduate students participating in this program will develop skills in microbiology, chemistry, hydrology, and environmental engineering. This type of multidisciplinary training is essential for addressing pollution problems confronting the Department of Defense.

Progress toward the training goals of the grant has been made as follows:

James B. Herrick, a fifth year Ph.D. student with major in the field of microbiology, and minor concentrations in genetics and biochemistry, has nearly completed his Ph.D. dissertation. Owing to the travel stipend provided by the AASERT grant, J.B. Herrick has attended the national American Society for Microbiology meeting in Las Vegas Nevada (May 1994) where he presented his research to his peers. The Ph.D. research (ASM abstract entitled "Genetic and Taxonomic Variation in Naphthalene Catabolic Bacterial Populations Native to a Coal Tar Waste-Contaminated Site") has utilized a combination of field and laboratory techniques to isolate and genetically characterize naphthalene-degrading bacteria from several locations within our coal-tar contaminated study area near Glen Falls, NY. Among the exciting aspects of J.B. Herrick's work are (1) DNA coding for naphthalene metabolism has been directly extracted from sediments from the field study

site (see Herrick *et al.*, 1993 and Moré et al. 1994; attached) and (2) genetic exchange among natural populations has been implicated because highly conserved nucleotide sequences of and portions of the gene that encodes the first catabolic enzyme in the naphthalene biodegradation pathway have been found to be distributed among a broad diversity of bacteria at our field study site.

J.B. Herrick's educational and career goals have been well served by the support provided by the AASERT program. Currently the laboratory research portion of J.B. Herrick's Ph.D. dissertation is nearly complete. Therefore the major emphasis is on writing. He will depart from Cornell University and the AASERT program in the Fall of 1994. Several post-doctoral positions have already been offered to Dr. Herrick - among them are posts at USEPA, USGS, General Electric, and Los Alamos National Laboratory. Dr. Herrick was also found to be the top candidate in a nation-wide faculty search at George Mason University in Virginia. Disappointingly, however, political and financial instabilities at that institution forced the termination of the position.

Karen G. Stuart has recently completed her first year of graduate training as an Environmental Toxicology major at Cornell. Prior to entering Cornell's graduate school program, Karen had majored in Biochemistry at the University of Wisconsin (see transcripts attached). Ms. Stuart had also spent one year in Africa as a Peace Corps volunteer, and 1 year as a laboratory technician in the Great Lakes Toxicology Research Laboratory at the State University of New York at Buffalo. The primary emphasis in the early curriculum of a new graduate student is coursework. As is evident from K.G. Stuart's summary of courses (attached) her academic performance has been excellent. In addition to attending classes during her first year of AASERT support, K.G. Stuart has worked in the laboratory setting with Drs. Madsen, Ghiorse and several environmental toxicology and microbiology graduate students. The projects K.G. Stuart has participated in have exposed her to DNA hybridization, analytical chemistry and microscopic techniques. These will assist in defining the approaches and ideas that will eventually constitute research contributing toward her Ph.D. degree. It is premature to define K.G. Stuart's Ph.D. research project - but it

certainly will combine the concepts and tools of environmental toxicology, molecular microbiology, analytical chemistry, with field work at our coal tar-contaminated study site.

Summary

During the first year of the AASERT grant entitled "Research Training for Understanding the Fate of Environmental Pollutants", two graduate students, J.B. Herrick and K.G. Stuart have been supported. J.B. Herrick, very near to the end of his graduate studies, with already two published articles to his name, has assimilated a unique combination of disciplines (microbiology, molecular biology, population genetics, field work, and microbial catabolism of environmental pollutants). His strong performance in the market place [top candidate in a nationally conducted faculty search at George Mason University, (with no postdoctoral training!)], attests primarily to Dr. Herrick's own abilities, but also reflects well on the education he has received at Cornell University. K.G. Stuart has the same promise as J.B. Herrick, however, her scientific career is still at a stage of early development.

93rd General Meeting, Las Vegas, Nevada

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Genetic and Taxonomic Variation in Naphthalene Catabolic Bacterial Populations Native to a Coal Tar Waste-Contaminated Site. J.B. HERRICK*, D.E. HINMAN, K.G. STUART, E.L. MADSEN, AND W.C. GHIORSE. Section of Microbiology, Cornell University, Ithaca, NY 14853.

We are investigating the processes and effects of adaptation of indigenous bacterial populations to polycyclic aromatic hydrocarbon contamination. We have randomly sampled and isolated naphthalene-mineralizing bacteria from a contaminated seep sediment which actively mineralizes naphthalene and from an adjacent soil which expresses naphthalene mineralization activity only after a significant lag period. Variation in naphthalene catabolism is being examined on three levels: the taxon, the gene, and the nucleotide. Isolates from the soil sample were closely related -- primarily members of the genus *Burkholdaria* -- but were unrelated to those from the contaminated seep. The presence of homologs to the *Pseudomonas putida* G7 naphthalene dioxygenase gene *nah*Ac and the regulatory gene *nah*R was assayed by PCR. Neither *nah*Ac nor *nah*R was detected by PCR in any of the soil isolates. However, 17 of 22 hybridized at low stringency to a *nah*Ac gene probe, indicating divergence has taken place from an ancestral gene. The degree of divergence, as judged by stringency of hybridization, differed markedly among the 17 *nah*Ac-positive isolates. Dioxygenase activity was found in all but one of the *nah*Ac-negative strains, as indicated by their ability to convert indole to indigo. Isolates from the contaminated sample were more diverse, including members of the γ- and β-proteobacteria as well as a group of gram-positives. Again, *nah*R was not found (by PCR) in any of the isolates. Eleven of twelve gram-negative strains isolated from the contaminated seep were found to possess PCR-amplifiable *nah*Ac; however, it was not amplifiable from any of the three gram-positive strains. Restriction maps of the *nah*Ac gene from six of the most taxonomically distant contaminated-sample isolates showed no variation whatsoever in sequence, though the restriction maps differed significantly from that of *P. putida* G7. This suggests that adaptation to coal-tar contamination by the culturable naphthalene-degraders at this site may have been partially mediated by the transfer of naphthalene genes from a common source.

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population genetics | naphthalene | adaptation

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Polymerase Chain Reaction Amplification of Naphthalene-Catabolic and 16S rRNA Gene Sequences from Indigenous Sediment Bacteria

JAMES B. HERRICK,[1] EUGENE L. MADSEN,[1] CARL A. BATT,[2] AND WILLIAM C. GHIORSE[1]*

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Received 27 August 1992/Accepted 22 December 1992

We report the amplification of bacterial genes from uninoculated surface and subsurface sediments by the polymerase chain reaction (PCR). PCR amplification of indigenous bacterial 16S ribosomal DNA genes was unsuccessful when subsurface sediment containing approximately 10^7 cells · g^{-1} was added directly to a PCR mixture. However, when 10 mg of sediment was inoculated with approximately 10^5 cells of *Pseudomonas putida* G7, the *nahAc* naphthalene dioxygenase gene characteristic of the *P. putida* G7 NAH7 plasmid was detected by PCR amplification. Southern blotting of the PCR amplification product improved sensitivity to 10^3 to 10^4 cells from samples inoculated with *P. putida* G7, but controls with no sediment added showed that the PCR was partially inhibited by the sediments. Lysozyme–sodium dodecyl sulfate–freeze-thaw DNA extraction was combined with gel electrophoretic partial purification in the presence of polyvinylpyrrolidone to render DNA from indigenous bacteria in surface or subsurface sediment samples amplifiable by PCR using eubacterial 16S ribosomal DNA primers. The *nahAc* gene could also be amplified from indigenous bacteria by using *nahAc*-specific primers when PCR conditions were modified by increasing *Taq* and primer concentrations. Restriction digests of the *nahAc* amplification products from surface and subsurface sediments revealed polymorphism relative to *P. putida* G7. The procedures for DNA extraction, purification, and PCR amplification described here demonstrate that the PCR is a potentially useful tool in studies of function- and taxon-specific DNA from indigenous microbial communities in sediment and groundwater environments.

Studies of the genetic composition and ecology of native bacterial populations traditionally have been constrained by their dependence on culture-based methods. It is an established dictum of microbial ecology that the majority of the bacterial species in nearly all microbial communities are unculturable (3), although a few notable exceptions have been reported (7, 36). Environmental microbiologists have therefore begun to explore the use of molecular methods to circumvent the bias of culture-dependent techniques. The analysis of extracted rRNA or ribosomal DNA (rDNA) has proven particularly fruitful for identifying bacteria in marine bacterioplankton (11, 34), terrestrial hot springs (37, 43, 45), and endosymbiotic associations (1).

In addition to taxonomic information, analysis of extracted nucleic acids can provide information about genetic variation in microbial populations which carry out environmentally important functions. For example, using DNA probes prepared from plasmid PJ4, which codes for 2,4-dichlorophenoxyacetic acid metabolism, Holben et al. (15, 16) found positive hybridization only with soil DNA extracted from one of two sites containing 2,4-dichlorophenoxyacetic acid mineralization activity. Also, Barkay et al. (6) demonstrated that four distinct probes for the *mer* operon hybridized to varying degrees with DNA extracted from pond and river water. When Barkay et al. (5) examined DNA extracted from mercury-resistant isolates, they found that a *mer* operon DNA probe and a more specific *merA* probe did not hybridize with the DNA under high stringency. Yet the DNA of all of the isolates hybridized with the *merA* probe at low stringency, indicating divergence of related sequences. Thus, the distribution and variation in genes encoding biodegradative and other microbial processes in natural environments are poorly understood.

A number of studies have shown that molecular approaches such as nucleic acid hybridization can be successfully applied in an environmental context (17, 31); however, these approaches are subject to their own methodological limitations. The techniques employed in many of these experiments may be selective or lack the sensitivity required to detect and analyze the genes of interest. Polymerase chain reaction (PCR) amplification of extracted DNA has been suggested as one method for overcoming these limitations in soils and sediments (32). Enzyme-dependent manipulations of nucleic acids, such as PCR, are hampered by the presence in extracted DNA of inhibitory natural substances which may not be removed by standard DNA purification techniques (39, 41, 42). Nonetheless, PCR has been employed to detect *Escherichia coli* (18, 41), a *Frankia* sp. (14), and a genetically engineered *Pseudomonas cepacia* (38) introduced into soils and sediments. Amplification of DNA sequences from indigenous bacterial populations, on the other hand, has proven to be much less tractable. Recently, PCR amplification of electrophoretically purified 16S rDNA sequences has been successful for indigenous soil bacteria (21); however, PCR amplification of catabolic genes present in native populations has not, to our knowledge, been reported.

The objectives of this study were (i) to develop methods for obtaining PCR-amplifiable DNA from indigenous microbial populations in surface and subsurface sediments and (ii) to apply the methodology to samples from a naphthalene-contaminated field site to determine whether naphthalene degradation gene sequences similar to those encoded on the

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TABLE 1. Description of samples used in this study

Sample designation	Sampling location	Date collected	Depth (m)	% Organic matter[a]	Naphthalene concn (μg/g)[b]	Mineralization potential[c]	
						Naphthalene	*p*-Hydroxybenzoate
Pristine	Pristine subsurface, vadose zone	April 1989	0.6	0.69	BD	0	52
Source	Contaminated subsurface at source of coal tar, saturated zone	June 1990	ca. 4	ND	33.8	45	70
Upgradient	Contaminated subsurface, 10 m downgradient from coal tar source, water table zone	September 1989	2.8	0.93	BD	19[d]	49[d]
Downgradient	Contaminated subsurface, 130 m downgradient from coal tar source, water table zone	September 1989	3.0	1.04	BD	36[d]	54[d]
Seep	Surface sediment in seep area within contaminant plume, 400 m downgradient from coal tar source	July 1991	0.1	20.6	7.0	55	50

[a] The percentage of organic matter for the seep sample was determined by high-temperature ignition (8); the percentages of organic matter for the other samples were determined by the loss-on-ignition method (24). ND, not determined.

[b] The naphthalene concentration was determined by gas chromatography or gas chromatography-mass spectrometry. Source and seep data are from reference 8, and all other data are from reference 24. BD, below detection.

[c] Cumulative percentage mineralized at 20°C in triplicate samples relative to poisoned controls. Source and seep samples were incubated for 10 days; all other samples were incubated for 14 days. The standard deviation for each value, as estimated by using 8 and 20 determinations, was 19% of the mean.

[d] Data from reference 25.

well-characterized *Pseudomonas putida* NAH7 plasmid were present in samples known to mineralize naphthalene. (Preliminary results were reported previously [13].)

MATERIALS AND METHODS

Site description, sampling, and microbiological characterization. Subsurface samples were obtained from a coal tar waste-contaminated site located in a forested sandy alluvium area in the northeastern United States. This site has been studied intensively (24–26), and buried sediments containing high concentrations of coal tar have recently been removed (10). However, downgradient portions of the site remain contaminated with soluble coal tar constituents. Approximately 400 m from the buried coal tar, contaminated groundwater seeps from the side of a hillslope. Table 1 provides a description of the samples employed in this study. Seep sediment samples were taken with a flamed trowel from approximately 10 cm below the surface of the litter layer. Coring and aseptic subcoring methods for subsurface samples upgradient from the seep, as well as methods for plate counts of viable bacteria and total microscopic acridine orange direct counts of bacteria, have been described (7, 25, 26, 36). Conversion of ^{14}C-labeled organic compounds to $^{14}CO_2$ was measured by standard methods (2, 23), modified as previously described (25, 26).

Bacterial strain. *P. putida* G7 was obtained from G. S. Sayler (Center for Environmental Biotechnology, University of Tennessee, Knoxville).

Primer and probe design. In *P. putida* G7 containing the NAH7 plasmid, the *nahAc* gene encodes the large subunit of the iron-sulfur protein component of naphthalene dioxygenase, the initial enzyme in the naphthalene catabolic pathway (35, 48). The positions of the 30-mer oligonucleotide PCR primers (noted below as nahAc1 and nahAc3) and the internal hybridization probe (noted below as nahAc2) were selected on the basis of two *nahAc* sequences, one from *P. putida* G7 (35) and one from *P. putida* NCIB 9816 (19). The coding regions of these two DNA sequences were found to be 96% identical by using the GCG sequence analysis software package (9). In addition, the amino acid and DNA sequences of the toluene dioxygenase of *P. putida* F1 (50) were compared with those of the *nahAc* gene. Two regions exhibiting amino acid conservation between the homologous aromatic dioxygenases and identical nucleotide sequences for the naphthalene dioxygenases were chosen as PCR primer locations. The sequence of PCR primer nahAc1 is 5′-GTT TGCAGCTATCACGGCTGGGGCTTCGGC-3′, corresponding to nucleotides 794 to 823 of the NCIB 9816 sequence (19), and the sequence of PCR primer nahAc3 is 5′-TTCGACAA TGGCGTAGGTCCAGACCTCGGT-3′, corresponding to nucleotides 1495 to 1466. The sequence of the internal oligonucleotide probe nahAc2 is 5′-GCTCGCGTGGAGAGCTTC CATGGCTTCATC-3′, corresponding to nucleotides 911 to 940. PCR amplification (33) with primers nahAc1 and nahAc3 results in a 701-bp product internal to the *nahAc* gene (the entire *nahAc* gene is 1,349 bp long).

The construction of oligomers for amplification of the 16S rRNA gene sequence was based upon regions conserved among all known eubacteria. Sequences were as described by Wilson et al. (46), our 5′ primer, designated 16SP-5, corresponded to P0mod, and our 3′ primer, 16SP-3, corresponded to PC5 of Wilson et al. (46). In addition, sequences containing restriction sites for cloning were synthesized as part of the primers but were not used in this study (44). The sequence of the 16SP-5 primer was 5′-ccgaattcgtcgacaacAG AGTTTGATCMTGG-3′ (linkers containing restriction sites for *Eco*RI and *Sal*I are indicated in lowercase letters; M denotes A or C). The sequence of 16SP-3 was 5′-cccgggatc caagcttTACCTTGTTACGACTT-3′ (lowercase letters indicate restriction site linkers for *Hin*dIII, *Bam*HI, and *Xma*I). These primers permit amplification of nearly the entire 1.5-kb 16S rDNA sequence (44).

Direct PCR from sediment. *P. putida* G7 was grown overnight at 30°C in 5% PTYG liquid medium (4), centrifuged, and resuspended in 15 mM sodium phosphate buffer (pH 7.0), and the suspension was held at room temperature for 2 h to deplete nutrient reserves. The cell suspension was then centrifuged again and resuspended in distilled deionized water (ddH_2O). The suspension was diluted as necessary

with ddH_2O before addition to sediment. Portions (10 mg) of nonsterile vadose zone sediment taken from the pristine borehole (Table 1) were inoculated with various concentrations of *P. putida* G7 in 2 μl of ddH_2O. Control tubes contained inoculated cells only (no sediment; positive control), sediment only, or PCR reagents only (negative controls). Inoculated samples were incubated at room temperature for 30 min. A 10-μl portion of detergent lysis solution (1% Tween 20, 5% Triton X-100, 10 mM Tris-HCl, 1 mM Na_2 EDTA [pH 8.0]) was added, and then the tubes were heated for 10 min at 85°C. Then 88 μl of PCR cocktail and 3 drops of light mineral oil were added directly to the sample tubes. The final reaction concentrations were 1 μM for each primer, 50 μM for each deoxynucleoside triphosphate, and 5 U for *Taq* polymerase (Promega Corp., Madison, Wis.) in a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.01% bovine serum albumin, and 0.05% Tween 20. The PCR was then carried out for 50 cycles consisting of 94°C (1 min), 55°C (2 min), and 72°C (3 min), with a 5-min extension at 72°C in the last cycle. All amplifications were performed on a Hybaid thermal cycler (Teddington, Middlesex, United Kingdom). Amplification products were electrophoresed in a 0.7% agarose gel and stained with ethidium bromide by standard techniques (30).

Extraction, purification, and PCR amplification of DNA. DNA was extracted from surface and subsurface samples by a modification of the method of Tsai and Olson (40). Briefly, 1 g (wet weight) of each sample was washed two or three times in 100 mM sodium phosphate buffer (pH 8.0) and then incubated in lysozyme solution (150 mM NaCl, 100 mM Na_2 EDTA [pH 8.0], 15 mg of lysozyme per ml) at 37°C for 2 h. A sodium dodecyl sulfate (SDS) solution (100 mM NaCl, 500 mM Tris-HCl [pH 8.0], 10% SDS) was added, and the reaction mixture was mixed well and incubated at room temperature for 5 min and then alternately frozen solid in liquid nitrogen and thawed in a 65°C water bath three times. An ammonium acetate solution (7.5 M) was added to achieve a concentration of 2.5 M, and the lysate was vigorously vortexed and then centrifuged at 6,000 × *g* for 10 min. The supernatant was transferred to a new tube and precipitated with ethanol and 20 μg of glycogen (molecular grade; Boehringer Mannheim, Indianapolis, Ind.) per ml. After drying, the precipitate was resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 7.6).

The extracted DNA was loaded into a 1% low-melting-point agarose gel (SeaPlaque GTG; FMC, Rockland, Maine) for purification. The gel was supplemented with 2% polyvinylpyrrolidone (Sigma) to aid in the separation of humic compounds from nucleic acids (39). Electrophoresis was carried out in 1 × Tris-acetate-EDTA (30) at a constant voltage of 5 V/cm for 90 min at 4°C. The gel was stained for 30 min in 1 × TAE containing 0.5 μg of ethidium bromide per ml. The resulting DNA band was located under UV illumination, excised from the gel, and melted at 60°C for 10 min, and 1 to 5 μl was added directly to the PCR mixture or, in the case of the seep-extracted DNA-agarose, diluted (1:100) in ddH_2O before addition to the PCR mixture. A band-sized piece of the gel was also excised from an empty lane at approximately the same position as the extracted DNA for use as a negative control. PCR was carried out as described above, but with primer concentrations of 0.5 μM, for 30 cycles consisting of 94°C (5 min), 42°C (30 s), and 72°C (4 min), with a 5-min denaturation step at 94°C in the first cycle and a 5-min extension at 72°C in the last cycle. The lower (42°C) annealing temperature was selected to allow amplification of *nahAc* genes that may have divergent base pair sequences.

Southern hybridization. For Southern blotting with ^{32}P-labeled probe, DNA was transferred after agarose gel electrophoresis to a nylon membrane (Magna Graph; MSI, Westborough, Mass.) and hybridized with the end-labeled nahAc2 oligonucleotide probe by standard procedures (30). Hybridization and washes were carried out under high-stringency conditions. For autoradiography, the blots were exposed to X-ray film at −70°C for 16 h.

Nonradioisotope digoxigenin labeling was also used for Southern hybridization with the *nahAc* probe. *P. putida* G7 cells were transferred from colonies on a 5% PTYG agar plate into 5 μl of ddH_2O and microwaved twice for 1 min at full power in a standard 700-W microwave oven (RCA) to lyse the cells. Digoxigenin labeling was carried out by a modification of the method of Lanzillo (20). A 20-μl portion of PCR cocktail was added to the distilled water cell lysate to give total concentrations of 0.5 μM for nahAc1 and nahAc3 primers, 35 μM for digoxigenin-dUTP (Boehringer Mannheim), 65 μM for dTTP, 100 μM for dATP, dCTP, and dGTP, and 0.5 U for *Taq* polymerase in the PCR buffer described above. A two-step PCR was carried out for 30 cycles consisting of 94°C (1 min) and 65°C (2 min), with a 3-min denaturation step at 94°C for the initial cycle and a 5-min extension at 65°C in the last cycle. A 10-μl portion of the reaction mixture was electrophoresed in 1% low-melting-point agarose, the single amplified probe band was excised, and the probe yield was quantified by direct detection according to the manufacturer's instructions (Boehringer Mannheim). The agarose-probe mixture was then heated at 95°C for 10 min to denature the probe. The purified digoxygenin-labeled probe was used in the standard hybridization procedure (30) after Southern transfer as described above. Labeled probe was added to the hybridization solution at a concentration of 10 mg of probe per ml of hybridization fluid. Hybridization and posthybridization washes were carried out under high-stringency conditions, and the bound probe was detected by chemiluminescent exposure (Lumi-Phos; Lumigen, Inc., Detroit, Mich.) of X-ray film for 1.5 min, according to the manufacturer's (Boehringer Mannheim) instructions.

Restriction digestion of amplified *nahA*. *nahA* sequences amplified from extracted DNA were electrophoresed in normal-melting-temperature agarose (high strength analytical grade; Bio-Rad, Richmond, Calif.), excised, and diluted 1:10 (source sample) or 1:100 (seep) in ddH_2O, and 5 μl was reamplified by using a two-step PCR as described above. The reamplified products were purified in a Centricon-100 microconcentrator (Amicon, Beverly, Mass.) by following the manufacturer's specifications, digested with restriction endonuclease, and electrophoresed in an 8% nondenaturing polyacrylamide gel.

RESULTS AND DISCUSSION

PCR amplification of *P. putida* genes from inoculated sediment. The primary objective of this investigation was to develop a rapid and reliable method for PCR amplification of bacterial genes native to our study site. Therefore, our initial experiments focused on amplification by PCR of 16S rDNA eubacterial sequences in the pristine sample (Table 1). At first, sediment was added directly to the PCR cocktail. Eubacterial primers for 16S rDNA were chosen for this experiment because these sequences should be abundant in most eubacterial communities (27). However, despite the

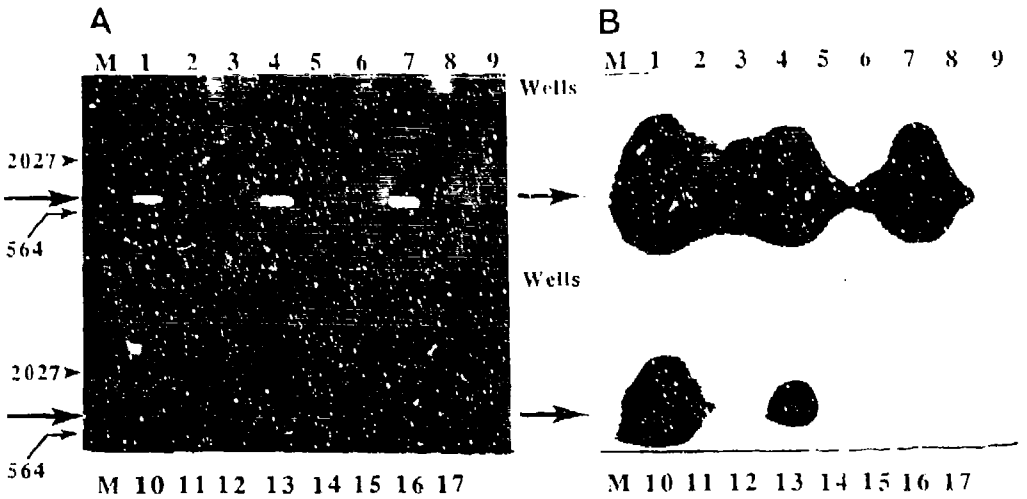

FIG. 1. PCR amplification of *nahAc* from 10 mg of pristine sediment inoculated with decreasing titers of *P. putida* G7 and added directly to the PCR reaction cocktail. (A) Ethidium bromide-stained agarose gel with amplified products loaded into two sets of wells in tandem. (B) Southern blot of gel shown in panel A hybridized with ^{32}P-labeled internal primer nahAc2, overexposed to increase sensitivity. Lanes: 1, 4, 7, 10, 13, and 16, no sediment; lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, and 17, sediment present; lanes 1 through 3, 1.6×10^5 CFU; lanes 4 through 6, 1.6×10^4 CFU; lanes 7 through 9, 1.6×10^3 CFU; lanes 10 through 12, 1.6×10^2 CFU; lanes 13 through 15, 1.6×10^1 CFU; lanes 16 and 17, no cells (negative controls); lanes M, lambda *Hin*dIII-*Eco*RI size marker. The lower broad band in some lanes is the so-called primer dimer artifact. The large arrows indicate the location of the 0.7-kb amplification product.

presence of 1.1×10^7 total bacteria per g and 2.0×10^5 culturable bacteria per g (25) and substantial *p*-hydroxybenzoate mineralization activity in this sediment (indicating the presence of active microbial populations) (Table 1), several attempts at direct PCR amplification of indigenous 16S rDNA genes in the sediment were unsuccessful. Parameters of the PCR protocol, such as thermal cycling times and temperatures, magnesium, *Taq* polymerase, and primer concentrations, lysis method, and amount of sediment, were modified without success. These negative results were not surprising in light of reports by other workers that materials native to the sediments, such as humic substances and mineral constituents, could be inhibitory to both restriction digestion (39) and *Taq* polymerase amplification (41) of extracted DNA.

PCR amplification was attempted after various numbers of washed whole *P. putida* G7 cells were mixed into the pristine sample. A 10-mg subsample of the seeded mixture was added directly to the PCR cocktail, and the naphthalene dioxygenase *nahAc* sequence was amplified directly, without cell or DNA removal or purification (Fig. 1). The *nahAc* product became visible when 1.6×10^5 CFU was inoculated (Fig. 1A, lanes 2 and 3). Southern analysis of the PCR products with the nahAc2 internal oligonucleotide probe increased the sensitivity to 10^3 to 10^4 CFU (Fig. 1B, lanes 5, 6, and 8). The PCR product was not observed in one replicate containing 1.6×10^3 CFU (Fig. 1B, lane 9), showing that variability existed in the samples at this level of sensitivity. On a per-gram basis, this degree of sensitivity is comparable to gene probing of inoculated, extracted, and unamplified total soil DNA (32). Our PCR sensitivity did not approach the 1-cell $\cdot$ g^{-1} limit reported by Steffan and Atlas (38). However, these authors used large (uninoculated) soil samples and laborious DNA extraction and gradient centrifugation techniques prior to PCR amplification.

We conclude from these results that the indigenous cells were in a different physical, chemical, or physiological state than cells added to the sediment in our experiment. Thus, the extraction of DNA from indigenous cells is a probable limiting factor for the PCR. This conclusion can be drawn only by assuming that the PCR amplification characteristics of the 16S primers were at least as sensitive as those of the *nahAc* primers. This is probably a conservative assumption because of the multiple copy number of 16S rRNA genes in bacteria (27). It is also evident in Fig. 1 that the sediment itself was inhibitory to the PCR. A strong hybridization signal was visible for the *nahAc* amplified without sediment from 1.6×10^1 CFU (Fig. 1B, lane 13), whereas the sensitivity of amplification with sediment was 2 to 3 orders of magnitude less (Fig. 1B, lane 8). The inhibition of the PCR may also have been caused by adsorption of released DNA to organic matter or clay in the sediment. Presumably, a compound which mimics the binding properties of DNA with sediment could be added to the sediment before the lysis step to ameliorate this problem. The addition of salmon sperm DNA, however, did not increase sensitivity (data not shown).

Extraction and purification of DNA. On the basis of the above results, it was apparent that DNA in the native bacteria was rendered less available for amplification than DNA from the added cells. Thus, methods for improving DNA extraction and purification were investigated. Crude DNA was extracted by a modification of the lysozyme–SDS-freeze-thaw protocol published by Tsai and Olson (40) (see Materials and Methods).

A significant problem with soil- and sediment-extracted DNA, particularly if it is to be subjected to enzymatic treatments such as PCR or restriction digestion, has been its association with inhibiting impurities, such as humic compounds (39, 41). Recent success in purifying DNA from *Frankia*-inoculated soil by agarose gel electrophoresis (14) raised the possibility that gel electrophoresis could render the DNA from indigenous sediment bacteria suitable for PCR amplification. Electrophoresis presumably strips off

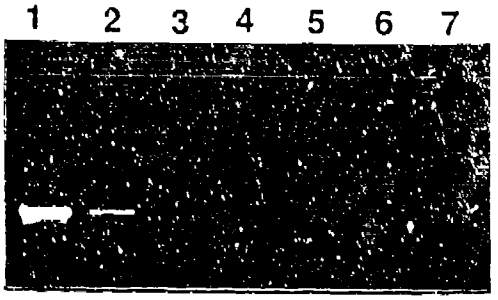

FIG. 2. Agarose gel electrophoresis of 16S rDNA amplification products obtained from polycyclic aromatic hydrocarbon-contaminated surface and subsurface sediments. PCR was carried out on DNA which had been extracted from aseptically collected field samples and partially purified by gel electrophoresis as described in Materials and Methods. Lanes 1, *P. putida* G7 cells inoculated from plate (positive control); lane 2, *P. putida* G7 DNA extracted and purified from whole cells by procedures used on sediments (positive control); lane 3, source sample; lane 4, seep sample; lane 5, downgradient sample; lane 6, upgradient sample; lane 7, purification gel only (negative control). See Table 1 for characteristics of samples.

humic compounds which may be bound tightly to nucleic acids and not easily removed, even by gradient centrifugation and column purification techniques. Young (49) has recently shown that the addition of polyvinylpyrrolidone to the agarose gel, which is thought to bind humic compounds, aids in the separation of DNA from humic components during electrophoresis.

The Hilger-Myrold (14) protocol for electrophoretic separation of DNA from humic contaminants originally called for electroelution of DNA from the gel and subsequent phenol-chloroform extraction of the eluted DNA. However, given the fact that the PCR was robust enough to occur even in the presence of unpurified sediment (Fig. 1), it seemed likely that separation of the electrophoresed DNA from molecular grade agarose before the PCR was implemented would be unnecessary. Therefore, the crude DNA lysate was electrophoretically purified in a low-melting-point agarose gel amended with polyvinylpyrrolidone. During electrophoresis of the extracted, unpurified DNA, brownish organic contaminants visibly separated from the genomic DNA band (data not shown). This band was then excised, and a portion of the agarose-DNA mixture was added directly to the PCR tube without further extraction from the gel.

PCR of indigenous 16S rDNA. PCR amplification of uninoculated 16S rDNA was successful when the DNA was extracted and purified by the methods described above. Figure 2 shows the gel electrophoresis of 16S rDNA sequences amplified from source, upgradient, downgradient, and seep samples. These four samples all contained microbial populations capable of mineralizing both *p*-hydroxybenzoate and naphthalene (Table 1). These samples were diverse in their physical characteristics; two were sandy subsurface sediments, one was a saturated zone sample heavily contaminated with polycyclic aromatic hydrocarbons, and one contained a high content of organic matter as well as polycyclic aromatic hydrocarbon contaminants (Table 1). Significant 16S rDNA amplification was obtained from the seep sample (lane 4), the source sample (lane 3), and the downgradient sample (lane 5). Only very weak amplification was observed from the upgradient borehole sample (Fig. 2, lane 6). Without DNA separation by electrophoresis, no 16S rDNA amplification was detected (data not shown). In a recent study by Liesack and Stackebrandt (21), gel electrophoresis of extracted DNA was also found to be necessary to permit amplification of 16S rDNA. Further experiments in our laboratory showed that PCR amplification of 16S rDNA was also possible after partial purification with a Prep-a-Gene DNA purification kit (Bio-Rad). However, the electrophoretic separation procedure described above was simpler and more reproducible than the Prep-a-Gene procedure.

PCR of extracted *nahAc* DNA. The above procedures for extracting, partially purifying, and amplifying 16S rDNA were used in initial attempts to amplify the *nahAc* sequence from DNA in source, upgradient, downgradient, and seep samples, all of which exhibited naphthalene mineralization activity (Table 1). When the thermal cycle program described above for direct sediment amplification of *nahAc* from inoculated *P. putida* G7 cells was used, amplification resulted in a smear of nonspecific products in each noncontrol lane of an ethidium bromide-stained agarose gel. We, therefore, employed the same program used successfully with the 16S rDNA primers. With this modification, reliable and specific amplification of *nahAc* from the source and seep samples was achieved (Fig. 3A, lanes 4 and 6). The identity of these *nahAc* sequences was confirmed by Southern hybridization under high-stringency conditions by using the labeled *nahAc* gene from *P. putida* G7 (Fig. 3B, lanes 4 and 6). Faint bands of amplified DNA from the remaining two subsurface samples running at approximately the same molecular weight as *nahAc* were detectable on the ethidium bromide-stained gel (Fig. 3A, lanes 5 and 7), and a longer exposure (3 versus 1.5 min) of the Southern blot shown in Fig. 3B revealed weak hybridization of *nahAc* to these bands (data not shown). However, additional attempts to amplify the *nahAc* genes in these samples did not give consistent results. Despite the presence of naphthalene mineralization activity in these samples, it is possible that the bacteria responsible for naphthalene catabolism have dioxygenase genes which are divergent from the *P. putida* G7 gene sequence, thus preventing amplification with the PCR primers used. Alternatively, the genes responsible for naphthalene mineralization in these bacteria may be entirely unrelated to *nahAc* because of (i) convergence of an unrelated sequence on the naphthalene dioxygenase function or (ii) the employment of an alternative pathway for naphthalene catabolism. In order to address some of these questions, we have begun to isolate naphthalene-mineralizing bacteria from this site and to screen them for the presence of amplifiable and unamplifiable *nah* genes and alternate pathways for naphthalene mineralization.

Restriction analysis of amplified *nahAc* sequences. Restriction and sequence analyses of individual genes can be useful in reconstructing relationships among those genes and the bacteria possessing them (12, 28, 47). However, sensitivity limitations may hamper the analysis of specific, low-abundance sequences in a heterologous mixture of extracted DNA. PCR can assist in overcoming such difficulties.

In an initial analysis of the amplified *nahAc* genes, the sequences were digested with restriction endonucleases known to cut *P. putida* G7 (NAH7) *nahAc*. To increase the yield of amplified *nahAc* for restriction analysis, the PCR product was electrophoresed in an agarose gel, excised, and directly reamplified in the agarose. Restriction digestions of the reamplified *nahAc* from the seep and source samples, compared with the restriction patterns of *P. putida* G7, are

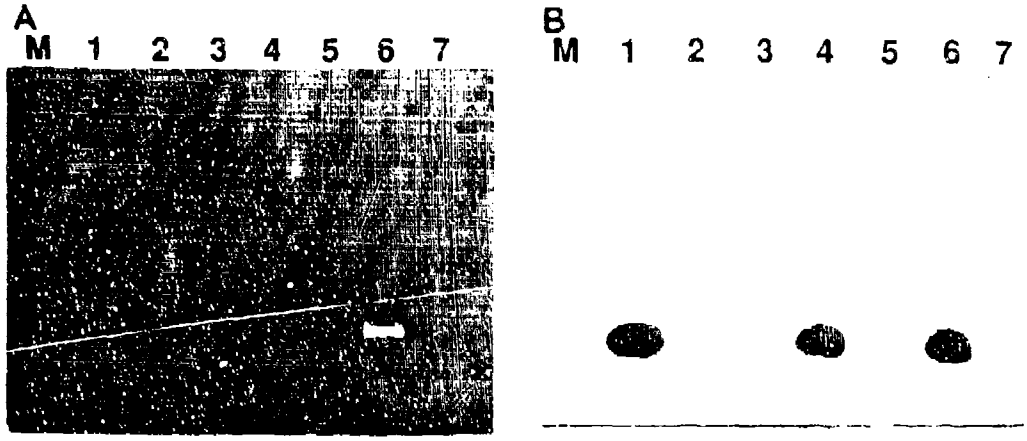

FIG. 3. Amplification of the *nahAc* naphthalene catabolic gene from native community bacterial DNA extracted and partially purified by gel electrophoresis from polycyclic aromatic hydrocarbon-contaminated surface and subsurface sediment. (A) Ethidium bromide-stained agarose gel. (B) Southern blot of gel shown in panel A hybridized to digoxigenin-labeled *nahAc* probe corresponding to the 701-bp *P. putida* G7 *nahAc* PCR product. Lanes M, lambda *Hin*dIII marker; lanes 1, *P. putida* G7 cells from plate (positive control); lanes 2, reagent only (negative control); lanes 3, purification gel only (negative control); lanes 4, source sample; lanes 5, downgradient sample; lanes 6, seep sample; lanes 7, upgradient sample.

shown in Fig. 4, *Hae*III and *Hin*fI restriction patterns for the source *nahAc* (Fig. 4, lanes 2 and 5) show the same size fragments as for *P. putida* G7, with additional fragments also visible. The presence of such fragments was presumably due to added restriction sites (polymorphism) in one or more of the sequences amplified. The restriction pattern of the seep *nahAc* sequences (lanes 3 and 6) was even more divergent from the pattern of *P. putida* G7 particularly at the *Hin*fI sites, which were both missing. There is no sequence variation at these restriction sites in the two *nahAc* sequences used to design the primers.

It should be recognized that the PCR products obtained in this study probably represent a mixture of *nahAc* sequences amplified from a number of different bacterial strains. PCR itself can be a selective process, randomly and possibly preferentially amplifying particular sequences or sequence families (29). Also, Liesack et al. (22) reported the assembly of a spurious hybrid sequence during PCR of a mixed culture

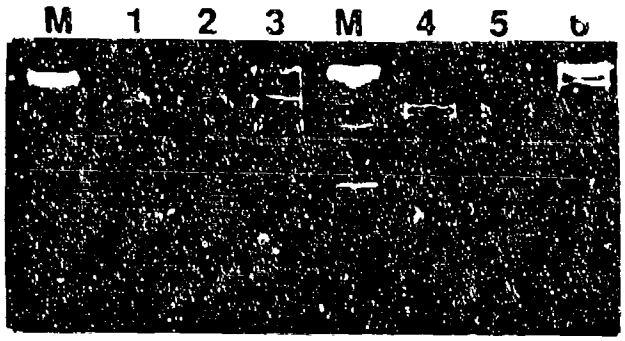

FIG. 4. Restriction endonuclease analysis of amplified, indigenous *nahAc* sequences compared with the *nahAc* sequence from the NAH7 plasmid of *P. putida* G7. Approximately equal amounts of DNA were loaded into all wells. Lane M, 123-bp size marker; lanes 1 and 4, *P. putida* G7 *nahAc*; lanes 2 and 5, source-derived *nahAc*; lanes 3 and 6, seep-derived *nahAc*; lanes 1 through 3, *Hae*III digested; lanes 4 through 6, *Hin*fI digested.

of barophilic bacteria. Thus, one must be cautious in drawing conclusions based upon analysis of mixed DNA sequences derived from PCR. However, the variation in the restriction patterns of extracted, amplified *nahAc* from the source and seep samples (Fig. 4) demonstrates that successful amplification was not simply an artifact (for example, a carryover of *P. putida* G7 *nahAc* PCR product). Also, the restriction digest results further confirm that bacteria with sequences similar to the NAH7 *nahAc* sequence are present at the field site.

Despite our conclusions about DNA sequences shared by *P. putida* G7 and bacterial community DNA at this study site, there is evidently a good deal of sequence variation in the amplified products. Deviation from *P. putida* G7 in the *nahAc* restriction patterns suggests that, although there is sufficient sequence similarity within the primer regions for amplification and for hybridization with an *nahAc* gene probe, there is notable sequence polymorphism within the *nahAc* genes distributed among the bacteria at the field site. This is particularly true for the seep sample. It is also possible that there is sequence divergence in naphthalene dioxygenase genes in populations at this field site which, because of the stringency of the PCR, was undetectable by these methods.

Successful PCR amplification of DNA from native microbial populations in environmental samples requires a sequence of events, including cell lysis, removal of the DNA from soil or sediment, denaturation of double-stranded DNA, annealing of single strands with primers, extension of primed DNA fragments with DNA polymerase, and many repeated cycles of denaturation, annealing, and extension. Clearly, impairment of any step in this complex chain will diminish yields. Little is known about the efficiency or sensitivity of each of the above steps as they apply to the methods described here. Moreover, no conclusions can be drawn about the abundance of amplifiable *nahAc*-containing cells or strains relative to those naphthalene degraders not possessing such sequences. However, this study has demonstrated that native 16S rDNA and *nahAc* sequences can be amplified from both surface and subsurface sediment sam-

ples and that amplifiable but divergent *nahAc* homologs are found in at least two regions of the field site. The subsequent analysis of PCR-amplified DNA from uncultured bacteria—whether by restriction digestion, DNA sequencing, or otherwise—will provide insights into the study of bacterial populations native to sediments and other terrestrial habitats.

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Quantitative Cell Lysis of Indigenous Microorganisms and Rapid Extraction of Microbial DNA from Sediment

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This study reports improvements in two of the key steps, lysis of indigenous cells and DNA purification, required for achieving a rapid nonselective protocol for extracting nucleic acids directly from sodium dodecyl sulfate (SDS)-treated sediment rich in organic matter. Incorporation of bead-mill homogenization into the DNA extraction procedure doubled the densitometrically determined DNA yield (11.8 μg of DNA · g [dry weight] of sediment^{-1}) relative to incorporation of three cycles of freezing and thawing (5.2 μg of DNA · g [dry weight] of sediment^{-1}). The improved DNA extraction efficiency was attributed to increased cell lysis, measured by viable counts of sediment microorganisms which showed that 2 and 8%, respectively, survived the bead-mill homogenization and freeze-thaw procedures. Corresponding measurements of suspensions of viable *Bacillus* endospores demonstrated that 2 and 94% of the initial number survived. Conventional, laser scanning epifluorescence phase-contrast, and differential interference-contrast microscopy revealed that small coccoid bacterial cells (1.2 to 0.3 μm long) were left intact after combined SDS and bead-mill homogenization of sediment samples. Estimates of the residual fraction of the fluorescently stained cell numbers indicated that 6% (2.2×10^8 cells · g [dry weight] of sediment^{-1}) of the original population (3.8×10^9 cells · g [dry weight] of sediment^{-1}) remained after treatment with SDS and bead-mill homogenization. Thus, lysis of total cells was less efficient than that of cells which could be cultured. The extracted DNA was used to successfully amplify *nahR*, the regulatory gene for naphthalene catabolism in *Pseudomonas putida* G7, by PCR. By scaling down the mass of sediment extracted to 0.5 g and by using gel purification and SpinBind DNA purification cartridges, the time required to extract DNA from whole sediment samples was reduced to 2 h.

Microbial ecologists, systematicists, and population geneticists have become increasingly interested in methods for complete, unbiased isolation of DNA (7, 9, 12, 16, 29, 30) and RNA (6, 8, 11, 19, 34, 36) from soils and sediments because such procedures promise to make the genomes of uncultured indigenous microorganisms available for molecular analysis. The ideal (2, 35, 36) is to circumvent the biases implicit in culture-based procedures by directly accessing the genes of naturally occurring microbial communities. But achieving this ideal requires overcoming a variety of interferences that diminish the quality, yield, and diversity of extracted nucleic acids. These interferences raise questions about the completeness of nucleic acid extraction, and about the representativeness of results based on the procedures.

The popular direct lysis approach to DNA extraction and purification (24) may be dissected into the following conceptual steps: (i) washing the material to remove soluble components that may impair manipulation of subsequently isolated DNA; (ii) disruption of cells in the material to release DNA or RNA from the cells; (iii) separation of the DNA or RNA from solids; and (iv) isolation and purification of the released DNA or RNA so that it can be used in various molecular procedures (i.e., PCR, digestion by restriction enzymes, hybridization reactions, or sequencing). A variety of methods integrating most or all of these steps have been published (7, 12, 20, 22, 28, 29, 31), yet, no study has demonstrated that the DNA or RNA was extracted from soil or sediment completely. Nor have criteria for complete extraction of DNA or RNA from native soil and sediment communities been established.

Procedures for lysis of microbial cells in soils and sediments have relied on one or more of the following treatments: lysozyme, heat, proteinase K, sodium dodecyl sulfate (SDS), achromopeptidase, hot phenol, guanidine thiocyanate, pronase, acetone, Sarkosyl, EDTA, freeze-thaw cycles, freeze-boil cycles, sonication, bead-mill homogenization, microwave heating, and mortar mill grinding. Ogram et al. (20) reported that a combination of SDS (incubated at 70°C) and bead-mill homogenization achieved a 90% lysis efficiency for cells native to marine and freshwater sediments, as determined by microscopic counts. Tsai and Olson (31) reported that an EDTA-lysozyme treatment followed by three freeze-thaw cycles reduced microscopic counts of cells added to sediment and subsoil samples by 95%. Similarly, Picard et al. (22) reported that three sonication microwave-thermal shock cycles achieved complete lysis of *Streptomyces* spores. More recently, Erb and Wagner-Döbler (7), using microscopic counts of two bacterial strains added to sterile sediments, concluded that six SDS, freeze-thaw treatments led to 99% lysis efficiency. While all of these reports were based on microscopic observations, descriptions of surviving-cell size distribution and morphology have yet to be presented. Furthermore, general criteria for lysis efficiency of microorganisms native to sediments have yet to be established. In this regard, several investigators (7, 22, 31) have made the questionable assumption that test microorganisms added to sediments were valid surrogates for native cells.

The rationale for the use of a lytic procedure is clear: complete disruption of cellular structure and release of nucleic acids is the objective. A goal of our research was to better understand the effectiveness of cell lysis procedures by deter-

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mining their effects on the diverse assemblage of cells in native microbial communities. In this investigation, we compared the effects of two of the most widely used physical lysis procedures, cycles of freezing and thawing and bead-mill homogenization, on DNA yield and viable-cell plate counts. Total counts and microscopic observations of acridine orange-stained samples were also used as criteria for lysis effectiveness. Finally, we simplified the protocol for extraction and purification of DNA from the sediment.

MATERIALS AND METHODS

Sediment samples. Sediment samples were obtained aseptically from a coal tar-contaminated site near South Glens Falls, New York. Sample characteristics and other details of the site have been described previously (12, 17, 18). Approximately 35 years ago, coal tar was buried in a single depositional event, and since that time, groundwater flow has distributed soluble coal tar constituents in a narrow contaminant plume through sandy subsurface sediments. The contaminated water, which contains naphthalene and phenanthrene, emerges in an organic matter-rich seep area at the foot of a hill slope, 400 m down-gradient from the original coal tar deposit. The methods described here primarily utilized the seep sediment, in which organic matter content was approximately 20% and the water content was approximately 50% (12). Other sandier sediments (approximately 1% organic matter and 20% water) were also used in this study; these subsurface sediments, designated "source," "upgradient," and "downgradient," were obtained from boreholes at the field site along a midline transect of the plume of groundwater contaminants (17, 18). In samples from source, upgradient, and downgradient locations, the concentrations of polycyclic aromatic compounds, especially naphthalene and phenanthrene, gradually diminished. Storage of seep and subsurface samples (in presterilized screw-cap glass jars) was at 4°C for periods up to 1 and 3 years, respectively. Any changes in microbial populations that may have occurred during storage were immaterial for the purposes of this investigation.

Cell lysis. The following general lysis protocol was used in all experiments. Equal weights (either 0.25 or 0.5 g) of wet sediment and phosphate buffer (100 mM, pH 8 [23]) were added sequentially to 2-ml screw-cap polypropylene microcentrifuge tubes (Laboratory Products Sales, Inc., Rochester, N.Y.) containing 2.5 g of 0.1-mm-diameter zirconia/silica beads (BioSpec Products, Bartlesville, Okla.) previously sterilized by autoclaving for 50 min at 120°C and 15 lb/in^2. Next, 0.25 ml of a 10% SDS solution (SDS-Tris-NaCl: 100 mM NaCl–500 mM Tris, pH 8–10% SDS) was added; the final concentration of SDS was approximately 4%. Each tube was shaken at high speed for 5 or 10 min in a bead-mill homogenizing unit (BioSpec Mini-Bead Beater). The selection of bead size and the proportion of beads to cell suspension were determined by following guidelines for disrupting bacterial cells provided by the manufacturer. The tubes were removed from the bead-mill and centrifuged for 3 min at 12,000 × *g*.

To compare the lysis efficiency of the bead-mill homogenization and freeze-thaw procedures, the sediment was mixed by adding 3 g of sediment to 3 ml of phosphate buffer in a 15-ml plastic centrifuge tube and mixing for 2 min on a vortex mixer; 0.5 ml of the mixed suspension (equivalent to 0.25 g of sediment) was immediately distributed to the 2-ml microcentrifuge tubes with and without prior addition of 0.1-mm beads as described above. A 0.5-ml suspension of *Bacillus* endospores in the phosphate buffer was also added to microcentrifuge tubes with and without beads. Endospores were harvested from a culture of *Bacillus subtilis* CU 1065 (Section of Microbiology, Cornell University) by culturing the bacterium on 5% PTYG agar medium (4, 5) and allowing extensive (approximately 40 days) desiccation to occur at 22°C. The spores were harvested by flooding the plate with the phosphate buffer. Microscopic examination showed that 100% of the *Bacillus* cells in the suspension had sporulated. Each tube received 0.25 ml of the 10% SDS–Tris–NaCl solution. The SDS-containing suspensions of the spores or sediment were then subjected to two different lysis procedures. In the freeze-thaw procedure, samples were rapidly frozen by immersion in liquid nitrogen (2 min) and then thawed in a 65°C water bath (5 min); this freeze-thaw cycle was carried out three times. The bead-mill homogenization procedure was carried out for 5 min as described above, with or without beads added. In this case, lysis efficiency was evaluated by triplicate viable-cell plate counts on 5% PTYG agar medium and microscopically as described below. The results were confirmed in three separate experiments, though data from only one are reported here.

Microscopic evaluation of cell lysis. Intact sediment samples or samples treated with SDS and subjected to the lysis procedures were stained with 0.01% acridine orange and examined with either a Zeiss Standard 18 microscope under phase-contrast and epifluorescence viewing or a Zeiss laser scanning microscope (model LSM-10) equipped for fluorescence, phase, and differential interference contrast imaging under 488-nm light from an argon laser. The LSM-10 is configured such that a single field of view can be examined by conventional transmitted and epifluorescence illumination or by comparable laser-scanning illumination. Both microscopes are fitted with × 100 oil immersion objective lenses with numerical apertures of 1.3 or 1.4. An acridine orange direct count (AODC) agar-smear procedure (5, 10) was used to assess the extent of lysis of the endospores and enumerate the total number of cells in the sediment. The computerized imaging and analysis systems of the LSM-10 were used to document the size distribution of microbial cells surviving the various lytic procedures. In enumerating cells in the sediment prior to implementing lysis procedures, the average count and standard deviation were computed from duplicate smears prepared from three independent subsamples of the sediment as described previously (5). In lysis experiments, the same general procedure was followed, except that only one smear from each sample was examined. In one instance, the number of surviving cells was estimated from a wet mount of a known volume of sample under a 22-mm^2 coverslip.

DNA purification. The supernatant from the lysis treatment (150 to 250 μl) was mixed 5:2 with a volume of 7.5 M ammonium acetate, and a precipitate was allowed to form for 5 min at 4°C. Then, the tube was spun for 3 min at 12,000 × *g* and 150 μl of the supernatant was concentrated and partially purified with a SpinBind DNA extraction cartridge (FMC BioProducts, Rockland, Maine). In a SpinBind cartridge, the DNA binds to a microporous silica membrane in the presence of chaotropic salts; after washing, the DNA can be eluted with water. The units were used according to the manufacturer's instructions, except that an EDTA-free ethanol wash buffer was employed and the DNA was eluted with 30 μl of warm (60°C) deionized water. The eluted DNA was loaded onto a 1% agarose gel containing 0.3 μg of ethidium bromide · ml^{-1} and subjected to electrophoresis (4 V/cm) for 20 min in Tris-acetate-EDTA (TAE) buffer according to a standard protocol (3). The resulting DNA bands were cut out of the gel and purified with a SpinBind cartridge according to the manufacturer's instructions for extraction from an agarose gel.

Quantification of DNA. The concentration of DNA after the

TABLE 1. Effect of freeze-thaw treatment and bead-mill homogenization on survival of culturable sediment bacteria and *Bacillus* endospores

Sample	Treatment[a]	CFU (±SD) · g^{-1} or ml^{-1}[b]	Survival	Viable/total cell ratio (%)[c]
Sediment	None	$1.0 (\pm 0.2) \cdot 10^7$	100	0.3
	SDS + freeze-thaw	$7.8 (\pm 1.5) \cdot 10^5$	8	0.02
	SDS + 5-min bead-mill[d]	$1.5 (\pm 0.2) \cdot 10^5$	2	0.004
Endospores	None	$1.8 (\pm 0.1) \cdot 10^5$	100	ND[e]
	SDS + freeze-thaw	$1.7 (\pm 0.3) \cdot 10^5$	94	ND
	SDS + 5-min bead-mill	$3.5 (\pm 0.5) \cdot 10^3$	2	ND

[a] See Materials and Methods.
[b] CFU for sediment computed per gram dry weight; CFU for endospores computed per milliliter.
[c] Also see Table 2; AODC of untreated sample = $3.8 (\pm 0.3) \times 10^9$ cells · gdw^{-1}.
[d] When beads were omitted from the homogenization procedure, the postlysis CFU count was $0.5 (\pm 1.0) \cdot 10^6$ (65% survival).
[e] ND, not determined.

final purification step was measured by densitometry as follows: 3.0 μl of each sample and 4, 2, 1, and 0.5 μl of Lambda DNA standards (Promega, Madison, Wis.) cut with *Hin*dIII (New England Biolabs, Beverly, Mass.) were electrophoresed on a 1% agarose gel in TAE running buffer as described above. The gel was photographed under a Spectroline 302-nm UV transilluminator (model TR-302) as previously described (37). A negative image of the gel was produced with a Polaroid MP4 Land camera using Polaroid type 55 film. Bands on the negative were scanned with a laser densitometer (Helena Laboratories, Houston, Tex.), and the DNA was quantified by interpolation from a calibration curve prepared from the densities of Lambda-*Hin*dIII-cut DNA standards.

PCR amplification of extracted DNA. The suitability of the isolated DNA to undergo enzymatic amplification reactions was tested by a nested PCR protocol (15, 27), using primers for *nahR*, the regulatory gene in the naphthalene catabolism gene cluster encoded on the NAH7 plasmid of *Pseudomonas putida* G7 (39). The outer primer sequences were 5'AACTGCGT GACCTGGATTTAA3' and 5'CGCCGCCGGCTCGGCTG GTGT3', corresponding to nucleotides 152 to 172 and 1244 to 1224 (39) of the *nahR* gene. The inner primer sequences were 5'GCCGCGCATCTGGCCGAGCCCGTCACTTCGG3' and 5'CTGGAGGATGTGGCCAACGGCGGCGAAGTGC3', corresponding to nucleotides 343 to 373 and 1200 to 1170 of the gene. The final product was 828 bp long. Reagents and conditions for carrying out the PCR were as previously described (12), except that the inner and outer reactions were prepared under "hot-start" conditions, with the deoxynucleoside triphosphates added after the tubes were heated to 80°C. The outer reaction mixture included 2 μl of SpinBind-purified sample and was cycled 1 time at 95°C for 5 min; 5 times at 94°C for 2 min, 65°C for 1 min, and 72°C for 1 min; 25 times at 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s; and 1 time at 72°C for 5 min. For the inner amplification which followed, 5 μl of solution produced from the outer reaction mixture was used as template. Tubes were cycled 30 times at 95°C for 30 s and 70°C for 1 min and 1 time at 72°C for 5 min. The PCR products were detected by agarose gel electrophoresis in 1% agarose gels as described above. *P. putida* G7, used as a positive control in the PCR assay, was originally obtained from G. S. Sayler (University of Tennessee) and was grown at 30°C in 5% PTYG as previously described (12). Negative controls in the PCR assay were done with reagent only (i.e., no added DNA) and a blank derived from a peripheral piece of the DNA purification gel that was taken through purification and amplification procedures.

RESULTS

One indication of the effectiveness of cell lysis procedures is cell viability. Therefore, we measured the change in viable bacteria (CFU) before and after bead-mill homogenization and freeze-thawing as an indicator of the extent of lysis. The data in Table 1 show that the bead-mill homogenization (2% survival) was more effective than freeze-thawing (8% survival) in reducing CFU of SDS-treated sediment bacteria. The CFU data only accounted for 0.3 to 0.004% of the total microscopic counts (Table 1); therefore, the survival of bacteria after the two cell lysis procedures was also tested with endospores of *B. subtilis*. Because of their resistance to physical disruption, endospores can serve as a model for other resistant microbial structures. The ineffectiveness of the freeze-thaw procedure in reducing the viability of a suspension of *B. subtilis* endospores was striking (94% survival [Table 1]) relative to the bead-mill homogenization, after which 2% of endospores remained viable. Lack of viability corresponded to the physical disruption of cell walls after bead-mill homogenization (Fig. 1). Phase-contrast microscopy showed that the phase-dense, refractile spores were completely ruptured after bead-mill homogenization (Fig. 1). The usual bright green fluorescence characteristic of DNA stained with acridine orange was missing in the ruptured spores and, therefore, had been released into the solution.

To further confirm that the reduced viability (Table 1) and ruptured cells (Fig. 1) were indicative of an extracellular release of DNA, we measured the yield of DNA from 0.5 g of sediment extracted and purified by several variations of the above lysis procedures: 10-min bead-mill homogenization as described above; three freeze-thaw cycles; or 5 min of bead-mill homogenization followed by three freeze-thaw cycles and then another 5 min of bead-mill homogenization. Initial qualitative examination of the yields from these three lysis methods was accomplished via 1% agarose gel electrophoresis (data not shown). On the basis of the fluorescence of intercalated ethidium bromide, there was no clear visual difference between the two treatments that utilized bead-mill homogenization. This suggested that bead-mill homogenization, alone, was as effective as a combination of freeze-thaw treatment and bead-mill homogenization in releasing DNA. In contrast, the intensity of the DNA band resulting from the freeze-thaw treatment alone was dimmest, thus corroborating the lower lysis efficiency of this treatment relative to bead-mill homogenization (Table 1). The DNA from all three lysis preparations was then concentrated with a SpinBind cartridge, employed in this study to improve DNA recovery over ethanol precipitation-DNA resuspension procedures used earlier (12). A portion of each

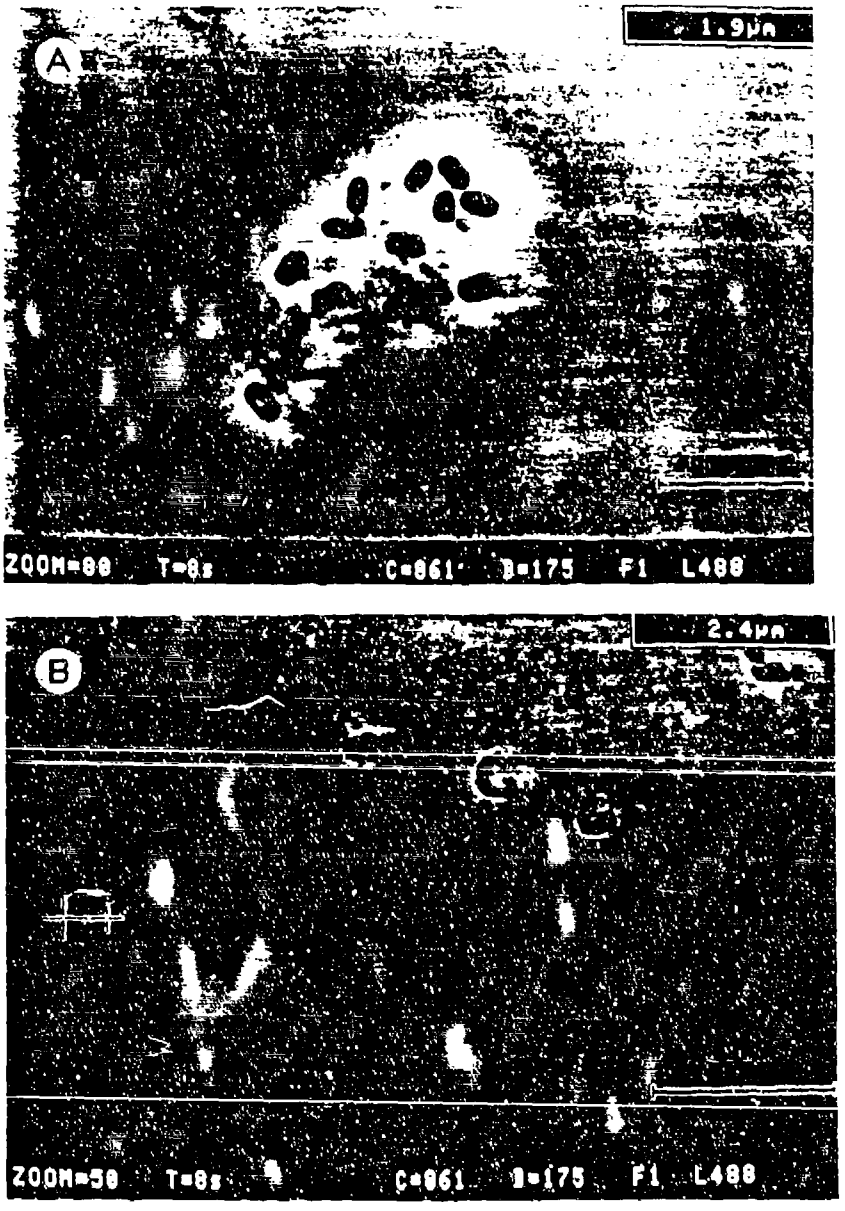

FIG. 1. Phase-contrast micrographs of *B. subtilis* spores before (A) and after (B) bead-mill homogenization. Dimensions in the upper righthand corner of each micrograph denote the distance between the crosses marking the ends of cells.

concentrated DNA preparation was next electrophoretically purified on a 1% agarose gel. Each DNA band was excised from the gel, processed a second time with the SpinBind cartridge, and visualized on an agarose gel, and then a negative image of each band was scanned with a laser densitometer to quantify the DNA. Yields from the bead-mill homogenization (alone), freeze-thaw treatment (alone), and the two lysis treatments combined were 11.8, 5.2, and 11.0 mg of DNA · g (dry weight) of sediment^{-1}, respectively. Thus, freeze-thaw treatment, alone, released one-half as much DNA as from the two bead-mill homogenization treatments, whose DNA yields were virtually indistinguishable.

Microscopic examination of the sediment provided an additional means of assessing the response of native microorganisms to lytic procedures. Because the data in Table 1 and the above DNA yields clearly demonstrated that freeze-thawing was a less effective cell lysis method than bead-mill homogenization procedures, only the latter and several variations (aimed at discerning the role of SDS in the procedure) were investigated. Table 2 reports the total bacterial numbers (AODC), approximate size distribution, and morphological diversity of microorganisms in sediments before and after SDS treatment, bead-mill homogenization, or both treatments. Prior to lysis, the sediment sample contained a rich and varied collection of both eukaryotic and prokaryotic cells which spanned a wide range of cell sizes (Table 2). In general, the various size fractions diminished as the severity of lytic procedures increased. The key observation shown in Table 2 is that

TABLE 2. Effect of bead-mill homogenization and SDS treatment on total bacterial numbers, bacterial size distribution, and morphological diversity in sediment

Treatment[a]	AODC (±SD) · gdw[-1][b]	AODC remaining (%)	Approx size range[c] (μm)	Morphological diversity[c]
Untreated	3.8 (±0.3) · 10[8][d]	100	>10–0.3	I
5-min bead-mill	1.9 (±0.5) · 10[8][e]	50	2.0–0.3	III
10-min bead-mill	1.0 (±0.2) · 10[8][e]	26	2.0–0.3	III
SDS	4.9 (±0.4) · 10[6]	13	5.0–0.3	II
SDS + 10-min bead-mill	2.2 (±1.6) · 10[8][f]	6	1.2–0.3	III

[a] See Materials and Methods; note that the SDS reagent was included in the bead-mill homogenization procedure reported in Table 1.

[b] gdw, grams (dry weight) of sediment.

[c] Size range and morphological diversity of fluorescent cells in at least 15 microscopic fields observed during AODC counting by conventional and laser scanning epifluorescence phase-contrast and differential interference-contrast microscopy. I, large and small filaments, rods, cocci, sarcina-like clusters of microcolonies. II, sarcina-like clusters and small coccoid cells only. III, small coccoid cells only.

[d] A factor of 380 greater than the untreated sample CFU described in Table 1.

[e] The AODC of this sample was estimated by determining the number of green fluorescent cells per ×1,000 field of 10 μl of a 1:8 diluted sample containing acridine orange spread under a 22-mm² coverslip.

[f] A factor of 1,467 greater than the SDS- and bead-mill-treated CFU described in Table 1.

approximately 6% [2.2 (±1.6) × 10^8 cells · g (dry weight) of sediment^{-1}] of the bacteria, mostly small coccoid cells in the sediment, were unaffected by the most severe treatment, bead-mill homogenization in the presence of SDS. The small cells which resisted lysis were observed by laser scanning epifluorescence microscopy (Fig. 2). It is important to note that the epifluorescence images are produced electronically in black and white by using a green analyzer filter and photomultiplier detector. Therefore, the degree of brightness of an object in these images was directly related to green fluorescence. It is also important to note that the ratio of viable to total counts (CFU/AODC) of the original sample was 0.3% before treatment (Table 1). After treatment with SDS and 10 min of bead-mill homogenization, the CFU/AODC ratio was 0.07% (inverse of factor in footnote *f* of Table 2). Thus, the net effect of these combined treatments was to lyse the larger cells that were more likely than the small cells to grow on the plate count medium.

PCR amplification of sediment-derived DNA. In addition to examining the efficacy of cell lysis procedures, this study also pursued the goal of achieving a rapid overall procedure for extracting and purifying DNA from sediment. By scaling down the total amount of sediment processed, from 1 (12) to 0.25 or 0.5 g, we were able to perform all of the above procedures in microcentrifuge tubes. This, in combination with utilization of SpinBind units, shortened the total processing time, from crude sediment to purified DNA, to approximately 2 h.

Many reports have shown that soil and sediments contain humic or other substances that may remain associated with extracted DNA, thus preventing its subsequent analysis (13, 29, 33). To determine if the DNA yielded from sediment samples was pure enough to allow subsequent molecular analysis, we performed a variety of tests. The first was designed to ascertain the effectiveness of gel electrophoresis in DNA purification. A 1-μl volume containing 45 *P. putida* G7 cells (determined by plate counts) was added to the PCR mixture along with 2 μl of sediment-derived DNA that had twice been passed through the SpinBind cartridges, with and without gel electrophoretic purification in between (the particular sediment subsample used here lacked amplifiable *nahR*). After completion of the nested PCR procedure on both preparations, *nahR* was amplified from *P. putida* G7 cells only with the electrophoretic purification (data not shown). Thus, we confirmed our previous results (12) indicating that the sediment contained PCR-inhibitory substances whose removal required a gel-electrophoretic purification step.

Prior work has shown that sediment samples from a variety of locations in our coal tar-contaminated field site contain genes homologous to *nahA* (12) and *nahR* (27). Using the DNA isolation and purification protocol described here, we repeatedly examined the quality of the DNA so obtained. Figure 3 shows the PCR products that resulted from four different sediment samples from our study site. Because PCR is sensitive both to concentrations of inhibitory substances and to the concentration of target DNA sequences, we amplified directly after the final elution from the SpinBind unit (Fig. 3, lanes 1, 3, 5, and 7) and after a 10-fold dilution (Fig. 3, lanes 2, 4, 6, and 8). The DNAs extracted from seep sediment (used to develop the protocols described in this study [Fig. 3, lanes 1 and 2]) and source sediment (Fig. 3, lanes 7 and 8) were susceptible to PCR amplification of the *nahR* gene, regardless of dilution. However, DNA preparations from the other sediment samples displayed differing responses to dilution. *nahR* was not amplified from the diluted upgradient sediment DNA (Fig. 3, lane 4)—possibly indicating a low titer of target DNA. In contrast, the DNA preparation from the downgradient sediment yielded a relatively weak amplification band in the undiluted sample Fig. 3, lane 5)—possibly indicating that the electrophoresis and SpinBind purification steps failed to completely remove substances inhibitory to the PCR. As an additional negative control treatment in the experiment whose results are shown in Fig. 3, a piece of the purification gel from outside the DNA bands was carried through the PCR procedure and failed to yield the amplified product (data not shown). When additional subsamples of the sediments used (Fig. 3) were repeatedly carried through the DNA extraction, purification, and PCR procedures, amplification of the *nahR* genes was not always consistent. This inconsistency was noted previously in the amplification of *nahAc* from the upgradient and downgradient samples (12). The reason for this variability is uncertain, but the variation may have been caused by heterogeneity inherent in the physical, chemical, and microbiological characteristics of field site-derived sediments.

DISCUSSION

This report has articulated the role of cell lysis as the first in a series of procedures required for achieving efficient, nonselective access to the genes in naturally occurring sediment microbial communities. But, perhaps more importantly, we have presented criteria for evaluating the effectiveness of the lysis step. These criteria were loss of cell viability, total DNA

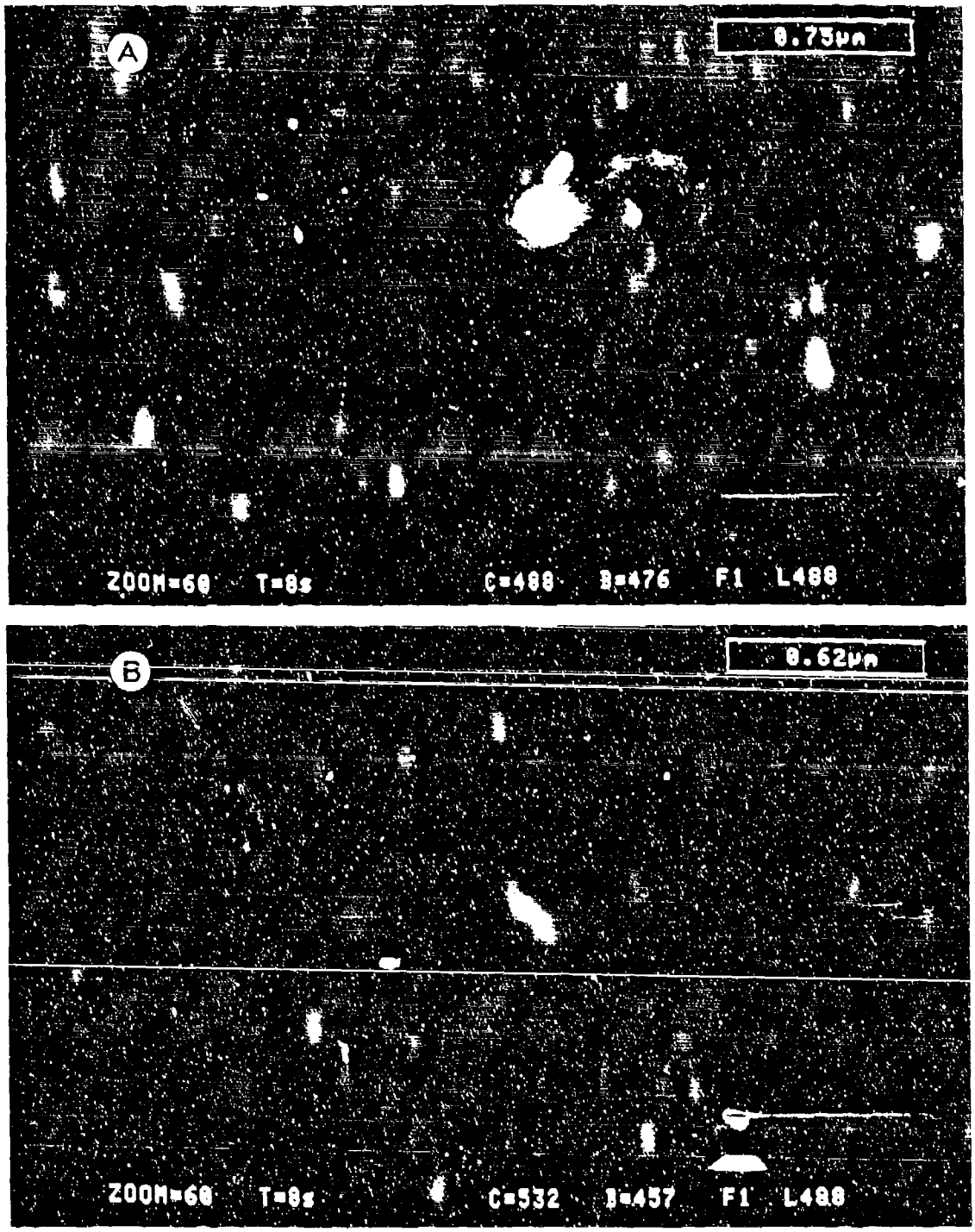

FIG. 2. Laser scanning epifluorescence micrographs of acridine orange-stained native cells in sediment before (A) and after (B) bead-mill homogenization. Note the presence of both large and small cells in the center of the prelysis micrograph. Only very small cells remained after lysis. Dimensions in the upper right-hand corner of each micrograph denote the distance between the crosses adjacent to cells or particles.

yield, and microscopic examination of sediment-derived cells for total direct counts and morphological diversity changes. By all four criteria, bead-mill homogenization was shown to be more efficient in lysing cells than freeze-thawing. Furthermore, the quality of the DNA subsequently extracted from the sediment was verified by PCR amplification of a native naphthalene catabolic gene.

Precedent has been set for using the behavior of an indicator microorganism, often seeded into sediments prior to determining the efficiency of lysis or DNA recovery, as a basis for

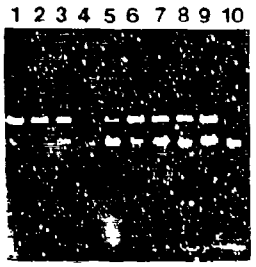

FIG. 3. Amplification of *nahR* from four sediment samples with nested primer PCR. For lanes 1, 3, 5, and 7, a 2-μl concentrated sample was used as a template for PCR amplification; and for lanes 2, 4, 6, and 8, a 2-μl 1:10 dilution of the same samples was used. Lanes: 1 and 2, seep sediment; 3 and 4, upgradient sediment; 5 and 6, downgradient sediment; 7 and 8, source sediment; 9, positive control (part of a *P. putida* G7 colony added to the PCR mixture); 10, PCR negative control (reagent only).

extrapolating to the behavior of indigenous cells (7, 22, 31). Similarly, in this study, viable counts of *Bacillus* endospores and native sediment bacteria were examined as a means for testing cell lysis procedures. A consistent proportion of surviving cells (2%) (Table 1) shared by the two very different microbial assemblages (total sediment microorganisms and *Bacillus* endospores) provided initial encouragement that DNA released from sediment by bead-mill homogenization would be completely representative of the sediment microbial community. However, the total viable counts derived from the sediment represented only 0.3% (prelysis) (Table 1) and 0.07% (postlysis) (Table 2, footnote *f*) of the total microscopic count. This total microscopic count necessarily included unknown proportions of nonviable but intact cells and both cultured and uncultured viable cells. Thus, an astonishingly large component of the sediment microbial community studied here was characterized only according to microscopically discernible traits such as cell size and morphology (Table 2). The SDS, bead-mill homogenization treatment disrupted indigenous cells in a biased manner by leaving the smallest size fraction (1.2 to 0.3 μm long) (Table 2; Fig. 2) intact. Until this resistant portion of the sediment microbial community can be lysed (perhaps by using smaller beads and additional chemical lytic agents), the ideal of accessing all of the indigenous genes will be thwarted. Furthermore, it is clearly unwise to use added indicator microorganisms, or even viable indigenous cells as a basis for drawing inferences about the susceptibility of the uncultured microbial community to cell lysis procedures.

Despite the fact that SDS, bead-mill homogenization failed to disrupt small cells native to the sediment, it is appropriate to use the data presented here to estimate total sediment DNA and the overall efficiency of the extraction procedure. If we presume that prokaryotes were the predominant reservoir of sediment DNA and that each of the $3.8 \cdot 10^9$ prokaryotic cells · g (dry weight) of sediment^{-1} contained a single stationary-phase genome weighing $5 \cdot 10^{-15}$ g (based on data for *Escherichia coli* [38]), then 1 g (dry weight) of the sediment contained 19 μg of DNA. This value agrees reasonably well with the total sediment DNA estimated by Ogram et al. (20) (27 μg · g dry weight^{-1}) and with the ranges of total soil DNA (20 to 50 μg · g dry weight^{-1}) reported by Picard et al. (22), Selenska and Klinginuller (26), and Steffan et al. (28). Factors contributing to variability in total DNA estimates include those imposed by different extraction methodologies, as well as microbiological idiosyncrasies of particular samples stemming from physiological influences such as soil or sediment type, climate, and the content of water, oxygen, and organic matter, etc. The DNA yielded when SDS, bead-mill homogenization was combined with the extraction protocol described here (11.8 μg · g [dry weight] of sediment^{-1}) represents 62% of the 19 μg of total theoretical DNA. Many of the assumptions contributing to this efficiency figure are uncertain; nonetheless, this estimated yield is reasonably high. It is perhaps remarkably high in light of the fact that much of the DNA from cells 1.2 to 0.3 μm in length was not released (Table 2) and that the steps subsequent to cell lysis (especially separation of the DNA from sediment particles) were not carefully scrutinized. Only after each step has been thoroughly examined and optimized can DNA extraction biases be reduced and efficiency be increased. It should be noted that even if an extraction efficiency of 99.9% were achieved, with 10^9 cells per g this would still leave 10^6 organisms per g unsampled. Thus, even when the lysis efficiency is relatively high, minor members of the community may remain intact and, consequently, their DNA may escape detection. Although there is no clear solution to this dilemma, we feel that continued striving towards the combination of unbiased genome sampling and enhanced sensitivity afforded by PCR may partially mitigate such detection limit problems.

PCR detection of genes native to sediment requires that the ratio of target sequence be high relative to accompanying sediment-derived materials that may inhibit the denaturation, annealing, and DNA synthesis stages of PCR (29, 32, 33). In this regard, optimal sensitivity for amplifying native genes can only be achieved by separating the DNA from inhibitory substances. Recently, Abbaszadegan et al. (1) have shown that Sephadex G-100 and Chelex 100 resins successfully removed PCR-inhibitory substances from groundwater concentrates. Perhaps ironically, nontarget DNA itself has also recently been shown to mask the PCR amplification of target sequences in low abundance (29). This study has confirmed that purification of DNA extracts is required for successful PCR amplification of indigenous genes (*nahR*). But even in such purportedly pure DNA preparations, lack of amplification in undiluted DNA extract (Fig. 3) suggested that inhibitory substances still remained in the mixture. The need to dilute DNA extracts prior to PCR amplification has been reported earlier for electrophoretic purification of DNA extracted from environmental samples (7, 22, 33), and it is the simultaneous dilution of the target sequence that may ultimately limit the sensitivity of the method.

Scale and its equivalent, sample size, are other issues to be considered in performing and interpreting experiments examining molecular characteristics of naturally occurring microbial communities. The small-scale (0.5 g) processing of sediment reported here substantially diminished the logistical and time constraints on DNA extraction. But facile processing of small samples raises questions about how accurately such small samples represent microbial communities as they occur in the landscape. Not enough is known about the chemical, physical, and microbiological spatial heterogeneity of soils (21) and sediments to allow data from 0.5-g samples to be the basis for extrapolation to larger (i.e., kilogram) or very large (i.e., landscape) scales. Moreover, the amplifiability of genes present in 0.5-g samples undoubtedly reflects the variable spatial distribution of both the target DNA sequence and sediment-derived substances that inhibit PCR (see the discussion above). Because these determinants for successful gene amplification may vary independently, interpreting the results of such assays may prove challenging.

Recently, Erb and Wagner (7) used DNA extraction and PCR amplification techniques to obtain a polychlorinated

biphenyl catabolic gene directly from a German freshwater sediment. A comparison of restriction digests failed to detect any divergence between a sediment-derived *bphABC* gene fragment and that of the type strain, *Pseudomonas* sp. strain LB400 (7). In contrast, by hybridizing DNA extracted from soils with a variety of gene probes Holben et al. (14) have recently demonstrated that the genetic basis in soil microbial communities for 2,4-dichlorophenoxyacetic acid catabolism was broader than that of plasmid pJP4. Similarly, we reported significant restriction fragment length polymorphism relative to *P. putida* G7 in the *nahAc* genes in DNA extracted from the same coal tar-contaminated field site examined here (12). In order to learn more about the distribution of related naphthalene catabolic gene sequences, procedures in this study utilized a different, nested set of oligonucleotide primers, specifically designed to amplify an 828-bp fragment of the *nahR* gene (27, 39). *nahR* is a member of the *lysR* family of regulatory genes that are widely distributed among gram-negative bacteria (25). Detection of *nahR* in the DNA extracted from the sediment provides two types of information. First, amplification of this gene allowed the quality of sediment-derived DNA to be evaluated. Because PCR amplification was possible, we concluded that the rapid extraction and purification procedures developed in this investigation were successful. But, perhaps more interestingly, detecting *nahR* in DNA extracted from this field study site lends additional momentum to ecological inquiries which utilize DNA sequence information from pure culture-derived functional genes to explore gene distribution and variation in nature.

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	William C. Ghiorse (Chair)	Microbiology
	Joseph M. Calvo	Genetics
	Charles F. Aquadro	Evolutionary Biology

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B Exam:

Date Degree Awarded:

Semester:	**Courses:**				
Summer 1989	Priciples of Biochem	BioS	331	4	B+
Fall 1989	Microbial Genetics	BioS	485	2	S
Spring 1990	Soil Microbiol., Lec	Agron	476	3	A-
	Bacterial Diversity	Micro	694	3	A+
Fall 1990	Population Genetics	BioS	481	4	B-
	Bacterial Cytology Lab	Micro	453	1	A-
Spring 1991	Survey of Cell Biology	BioS	432	3	A
All semesters	Microbiology Seminar	BioS	797	0	A
	Grad Res Sem in Micro	BioS	798	1	SX
First 3 semesters	Grad Sem Microbiol	BioS	799	1	SX
Fall 1990	Current Gen/Dev Topics	BioS	783	1	SX
Spring 1991	Adv Topics in Popul. Gen	BioS	684	2	S
Fall 1992	Hazardous Waste Toxicology	Tox	680	.5	SX
	Current Topics in Env Tox	Tox	698	1	NGR
	Biodegradation/Bioremediation	Tox	698	1	SX
Spring 1993	Hazardous Wsaste Toxicology	Tox	680	.5	SX

TA Semesters: Fall 1989, Spring 1990; Fall 1993; Spring 1994

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Eugene L. Madsen — Microbiology

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B Exam:

Date Degree Awarded:

Semester:	Courses:				
Fall 1993	Intro Chem & Environ Toxicol	Tox	610	3	A+
	Topics in Evol. Toxicol	BioS	400	3	A-
	Bacterial Diversity	BioS	415	3	A-
Spring 1994	Aquatic Chemistry	CEE	654	3	A
	Ecotoxicology	Tox	607	3	A-
	Immunotoxicology	Tox	611	1	A
All semesters	Seminar in Toxicology	Tox	702	1	S
Fall 1993	Safety Eval in Publ Hlth	Tox	660	2	S
Spring 1994	Hazardous Waste Toxicology	Tox	680	.5	A

TA Semesters:

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			CRS	GR	PTS
09/03/85		ENGLISH 101 OR BASIC COMPOSITION REQUIREMENT SATISFIED			
09/03/85		CEEB CREDIT			
ENGLISH	XXX	(AP ENGL LIT & COMPOSITION)	3.0		
HISTORY	101	AMER HIST TO CIVIL WAR ERA	3.0		
HISTORY	102	AMER HIST-CIVIL WAR-PRESENT	3.0		
HISTORY	119	MODERN EUROPE 1500-1815	3.0		
HISTORY	120	EUROPE&MOD WORLD 1815-ON	3.0		
		TOTAL DEGREE CREDITS	15		
09/03/85		ADMITTED TO THE LETTERS AND SCIENCE HONORS PROGRAM			
1-1985-86		BA 1 UNDERGRAD			
CHEM	109	GEN & ANALYTICAL CHEM I	H5	C	10.0
FRENCH	227	CONVERSTN& COMPOSITN-INTMED	3	AB	10.5
LIT TRAN	201	19 & 20TH C RUSSIAN LIT I	3	A	12.0
MATH	221	CALCULUS&ANALYTIC GEOMETRY	5	BC	12.5
SUM: DEG CR		16 GPA CR 16 GPA 2.812	16		45.0
02/27/86		CREDIT BY DEPARTMENTAL RECOMMENDATION			
FRENCH	101	FIRST SEMESTER FRENCH	4.0		
FRENCH	102	SECOND SEMESTER FRENCH	4.0		
FRENCH	203	THIRD SEMESTER FRENCH	4.0		
FRENCH	204	FOURTH SEMESTER FRENCH	4.0		
		TOTAL DEGREE CREDITS	16		
2-1985-86		BA 2 UNDERGRAD			
BACT	101	GENERAL MICROBIOLOGY	3	A	12.0
COM ARTS	181	ELEMENTS OF SPEECH	H3	AB	10.5
FRENCH	222	INTRO FRENCH LIT-19 & 20 C	H3	AB	10.5
MATH	222	CALCULUS&ANALYTIC GEOMETRY	5	AB	17.5
MUS PERF	201	ELEM/INTMED PIANO	2	AB	7.0
SUM: DEG CR		16 GPA CR 16 GPA 3.593	16		57.5
1-1986-87		BA 3 UNDERGRAD			
CHEM	343	INTRO ORGANIC CHEMISTRY	3	A	12.0
FRENCH	221	INTRO-MIDDLE AGES TO REVOL	H3	A	12.0
MUSIC	054	CHORALE	1	A	4.0
POLI SCI	336	SOVIET FOREIGN POLICY	4	B	12.0
PSYCH	202	INTRODUCTION TO PSYCHOLOGY	3	A	12.0
SOC	210	INTRODUCTION TO SOCIOLOGY	3	A	12.0
SUM: DEG CR		17 GPA CR 17 GPA 3.764	17		64.0
12/21/86		DEANS LIST			

			CRS	GR	PT
2-1986-87		BA 3 UNDERGRAD			
CHEM	344	INTRO ORGANIC CHEMISTRY LAB	2	A	8.
CHEM	345	INTMED ORGANIC CHEMISTRY	3	A	12.
FRENCH	332	FRENCH LIT-19TH CENTURY	H3	A	12.
STAT	224	ELEMENTARY STAT ANALYSIS	3	A	12.
ZOOLOGY	152	INTRODUCTORY BIOLOGY	5	B	15.
SUM: DEG CR		16 GPA CR 16 GPA 3.687	16		59.
08/23/87		TRANSFER CREDITS			
PHYSICS	201	GENERAL PHYSICS			
PHYSICS	202	GENERAL PHYSICS			
		TOTAL DEGREE CRED			
1-1987-88		BA 4 UNDERGRAD			
BIOCHEM	501	INTRODUCTION-BIOCHEMIST			
CHEM	346	INTMED ORGANIC CHEM LAB			
FRENCH	454	AFRICAN PROSE&POETRY FR			
FRENCH	547	HIST DE LA CIV FRANCAI			
GENETICS	466	GENERAL GENETICS			
SUM: DEG CR		14 GPA CR 14			
12/23/87		DEANS LIST			
2-1987-88		BA 4 UNDERGRAD			
BACT	320	ADVANCED GEN BACTE			
CHEM	110	GEN & ANALYTICAL CHEM			
FRENCH	548	HIST DE LA CIV FRANCA			
MUSIC	052	WOMENS CHORUS			
SUM: DEG CR		13 GPA CR			
1-1988-89		BA 4 UNDERGRAD			
BIOCHEM	651	GENERAL BIOCHEMISTR			
BIOCHEM	691	SENIOR THESIS			8
CHEM	565	PHYSICAL CHEMISTRY	4	A	16
FRENCH	228	CONVERSTN & COMPOSTN INTMD	3	AB	10
FRENCH	455	FRENCH FILM	3	AB	10
SUM: DEG CR		14 GPA CR 14 GPA			
2-1988-89		BA 4 UNDERGRAD			
BIOCHEM	511	UNDERGRADUATE SEMINAR	2	A	8.
BIOCHEM	692	SENIOR THESIS	1	S	0.
BIOCHEM	699	SPECIAL PROBLEMS	1	A	4.
CHEM	563	PHYSICAL CHEMISTRY LAB	1	AB	3.
ENV TOX	606	COLLOQUIUM IN ENV TOX	3	AB	10.
FRENCH	333	20TH CENTURY FRENCH LIT	3	A	12.
FRENCH	548	QUESTIONS DE CIV FRANCAISE	AU	S	0.
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ADV STG CREDITS	41	
CUM DEGREE CREDITS	159	
CUM GPA CREDITS	117	
CUM GRADE POINTS	424.0	
UNDERGRAD GPA	3.623	

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